2007. The Journal of Arachnology 35:278–292

THE UTILITY OF MOLECULAR MARKERS FROM NON-LETHAL DNA SAMPLES OF THE CITES II PROTECTED "TARANTULA" *BRACHYPELMA VAGANS* (ARANEAE, THERAPHOSIDAE)

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ABSTRACT. Tarantula spiders of the genus *Brachypelma* Simon 1891 are the only complete genus of arachnids protected from international trade under CITES law. To better understand the genetic cohesion of spiders within this genus, we evaluated multiple genetic fragments (totalling about 2200 bp) for their ability to recover population sub-structure among wild-caught *Brachypelma vagans* (Ausserer 1875) from Belize. We used a novel non-lethal method of tissue sampling, by inducing autospasy of the medial leg. This method allowed us to release wild-caught individuals of this protected species after DNA sampling. We used arachnid specific PCR primers to amplify targeted regions of *B. vagans* DNA, testing various combinations for consistency. We compared mitochondrial fragments from two populations of *B. vagans* (~50 km apart) for variation in mitochondrial 16S lrRNA (plus 5′ ND1), CO1, and the nuclear ITS-2 spacer. Both lrRNA-ND1 and CO1 provided congruent estimates of population subdivision, and indicated that lrRNA-ND1 contained the greatest variation. The nuclear ITS-2 was surprisingly short (193 bp) and relatively invariant across *B. vagans*. While both mitochondrial fragments appear suitable to elucidate population subdivision and historical processes in *B. vagans*, we suggest that mitochondrial markers may overestimate population division in *B. vagans*. We conclude that along with valuable inferences from mitochondrial regions, the characterization of population sub-structure in tarantula spiders will be enhanced by other estimates from alternate nuclear fragments.

Keywords: Belize, forced autospasy, molecular divergence

The "tarantula" family Theraphosidae (Araneae, Mygalomorphae) includes some of the most impressive spiders alive today. In particular, members of the genus *Brachypelma* are conspicuous members of the Mesoamerican invertebrate fauna, but are threatened by habitat destruction, road building, and illegal pet-trade collection (Cleva 1998; Locht et al. 1999). Over-collection of the red-knee tarantula *Brachypelma smithi* (F.O. Pickard-Cambridge 1897) for the pet-trade was a major factor that led to the blanket protection of the entire genus under CITES (Convention on International Trade in Endangered Species). To date, *Brachypelma* is the only genus of arachnids completely protected under CITES (Anonymous 1998). The anthropogenic pressures on natural populations of *Brachypelma* are exacerbated by high juvenile mortality rates (Baerg 1958) and low population densities (Yáñez & Floater 2000).

Conservation efforts to protect threatened arthropods can be most effectively focused with clarification of the genetic affinities among individuals (Joyce & Pullin 2003; Paquin & Hedin 2004). Here, we use three genetic regions to evaluate population subdivision in the red-rump tarantula *Brachypelma vagans* (Ausserer 1875). Unlike most *Brachypelma* species with narrow geographic ranges, *B. vagans* is unusually widespread (Locht et al. 1999; Striffler & Graminske 2003; West 2005). This species is found across much of the Yucatan peninsula from Mexico to Guatemala and perhaps Honduras, with minor morphological variation across the range (Smith 1994). The wide distribution of *B. vagans* compared to its congeners, and its sur-

vival in a wide variety of natural habitats (from peri-humid forest to savannah ecotypes) suggests that the species is, in reality, a low risk species for conservation priorities. Furthermore, *B. vagans* exploits areas extensively modified by human activity (Nicholas 2002; M'Rabet et al. 2005), showing a broad habitat tolerance unlike most other members of the genus, where the ecology may be easily disrupted by human activity (Locht et al. 1999; Yáñez & Floater 2000). In this context, the widespread *B. vagans* might provide a low-risk model against which to evaluate the population dynamics of more vulnerable *Brachypelma* species.

Most fossorial tarantulas, including all *Brachypelma* spp., have similar ecologies to *B. vagans*, and hence share population dynamics that can result in clustered spatial distribution and over-dispersion (Reichling 1999, 2003; Yáñez & Floater 2000; Shillington & McEwen 2006). Long-range gene flow is mediated by mature males, which migrate long-distances between aggregated populations of females (Shillington & Verrell 1997; Janowski-Bell & Horner 1999; Yáñez & Floater 2000). Exceptionally in *B. vagans*, recently hatched siblings show an unusual "group-dispersal" behavior (Reichling 2000, 2003; Nicholas 2002; Shillington & McEwen 2006). This unusual behavior, unknown in other species of *Brachypelma* may add to the relative persistence and wide geographic range of *B. vagans* compared to its congeners and might allow new populations (i.e., colonies) to establish from sibling groups that dispersed in unison. However, to our knowledge, genetic affinities have not been evaluated in any natural populations of fossorial tarantulas to date.

A problem for genetic studies of threatened taxa is the potential need to sacrifice valuable specimens (Lushai et al. 2000). This is not defensible with tarantulas due to their small population sizes, slow growth, and low reproductive success. We therefore evaluated a non-lethal technique for DNA tissue sampling, by inducing leg autospasy from live specimens (Longhorn 2001, 2002). To clarify the population sub-structure and gene flow in *Brachypelma vagans*, we then evaluated three genetic fragments for their ability to distinguish individuals from two populations in Belize. The combination of geographic and genealogical data (phylo-geographic analyses) has been widely used to infer the historical distribution and range movements of araneomorph spiders (Hedin 1997a, b; Masta 2000a; Hormiga et al. 2003; Ayoub & Riechert 2004; Paquin & Hedin 2004; Arnedo & Gillespie 2006; Murphy et al. 2006). Few studies of this type have been conducted with mygalomorph spiders (but see Bond et al. 2001, 2006; Bond 2004; Hendrixson & Bond 2005; Woodman et al. 2006), none of which focused on the tarantula family Theraphosidae.

We suggest that estimates of gene flow and population sub-structure from the widespread species *B. vagans* can help clarify the geographic distribution of other threatened *Brachypelma* species. Due to sex-biased dispersal patterns in fossorial theraphosids, we predict that mitochondrial markers might provide suitable resolution for population-level studies of theraphosids, and plausibly reveal a high degree of population sub-structure as in other mygalomorphs (e.g., Bond et al. 2001). By tracking the maternal lineage, we might expect mitochondrial markers to show minimal genetic differentiation among spiders within localized (spatially aggregated) populations and much greater differentiation across neighboring populations. In contrast, nuclear markers might be expected to show a greater degree of homogeneity across different levels of geographic sampling, due to the greater degree of admixture and gene flow mediated by long-range migration of mature males.

Here, we compare genetic variability of two mitochondrial fragments (16S lrRNA-ND1, and CO1) and a nuclear region (ITS-2 spacer) from *B. vagans* to define the variation in different genetic regions and evaluate population subdivision. The genetic sub-structure of *B. vagans* populations may be useful to contrast against other allied species with narrower geographic distributions. Clarifying the genetic affinities of any natural populations of theraphosid spiders, especially the genus *Brachypelma*, should facilitate attempts to maintain viable populations of the most threatened species and help ensure their long-term survival.

METHODS

Non-Lethal Tissue Sampling.—As a novel source of DNA, we removed a single medial leg from tarantulas by autospasy, inducing the voluntary fracture at the coxa-trochanter joint (Breene 1998; Johnson & Jakob 1999; Brau-

tigam & Persons 2003). We chose to remove a medial limb (III) as anterior legs (I–II) are used in sensory behaviors such as prey detection, while posterior legs (IV) are used defensively in brushing urticating hairs (Smith 1994). Initial trials of autospasy were conducted on captive theraphosids prior to field trials, mostly on other species of *Brachypelma* (n = 30). This allowed an evaluation of their survivorship in a controlled environment. Field trials were conducted on two populations of *B. vagans* in Southern Belize (48.2 km apart). Field caught spiders were lured from burrows at dusk during the primary foraging period by vibrating a grass stalk near the burrow entrance to simulate prey movements (Longhorn 2002; Nicholas 2002). We sampled several individuals (n = 14) in a grass clearing within a broadleaf forest near the *Las Cuevas* Research Station in the Chiquibul forest, Southern Cayo District, Belize (16°44′08″N, 88°59′20″W). The second field sample (n = 8) was from an open grassy area near Pooks Hill Lodge, Northern Cayo District, Belize (17°09′25″N, 88°51′13″W). Captured spiders were restrained by a sponge in a clear plastic container, and the femur of leg III grasped using forceps. The leg was then gently manipulated until voluntary rupture of the coxal apodeme was achieved and the isolated limb placed in absolute ethanol (stored at −20° C). The natural wound seal at the coxal stump was artificially enhanced with cosmetic nail hardener during which time spiders were placed in open containers to allow air-circulation (after Breene 1998). Spiders were released back to intact burrows within a few hours of the procedure (after confirming wound sealing). Where possible, burrows were revisited over successive days to assess the status of their occupant. All tissue samples were collected by permits from the Belize forestry department (CD/60/3/01 and CD/72/2/01) and transported under CITES permits (Export 001214; UK Import 237532). Tissue is stored at −80° C at the Natural History Museum (NHM), London, UK. We also collected a single voucher specimen from each locality, both confirmed as *B. vagans* by morphological evaluation (by Mr. A. Smith). Specimens were deposited together in the NHM arachnid collection under accession BMNH (E) 2003-148.

DNA extractions.—Under sterile conditions, ~1 g of muscle was taken from isolated legs of 22 *B. vagans* (wild caught from Belize) and a mature male *B. angustum* Valerio 1980 (captive-bred stock). Tissue was vacuum dried, then incubated at 37° C in the presence of 5 μl of 20 mg/ml proteinase K, 20 μl of 10% SDS, and 200 μl of TEN buffer (200 μl of 0.1 M Tris-HCl at pH 8.0, 100 mM NaCl, 10 mM Na_2EDTA). DNA was extracted with phenol:chloroform:isoamyl alcohol (24:24:1) and then chloroform:isoamyl alcohol (24:1), before precipitation with absolute ethanol: NaAc, then rinsed with 70% ethanol, and resuspensed in ddH_2O.

PCR primers.—For each targeted genetic fragment, multiple primers were tested for reliable PCR amplification in 50 μl reactions containing 1–2 μl of $MgCl_2$ (50 mM), 1 μl of dNTPs (10 mM), 1 μl of each primer (10 pmol), 5 μl buffer (10 ×), 0.2 μl (1 U) of *Taq* (Bioline), each made to volume with dd H_2O.

To amplify ~1-Kb of the mitochondrial lrRNA (16S) to ND1 (NADH1) we used LR-N-13398 (CGCCTGTTTAACAAAAACAT) (Simon et al. 1994, aka 16Sar) with NI-J-12261 (TCRTAAGAAATTATTTGA) (Hedin 1997a, b). In other spiders [except Mesothelae], this fragment includes a single tRNA (Leucine; CUN) and a short spacer region (Fig. 1, top). Fragments were amplified using a thermal cycle of 95° (120 s); 5 cycles of 95° (30 s), 46° (30 s), 72° (120 s); then 35 cycles of 95° (30 s), 48° (30 s), 72° (120 s); and 72° (10 min). We used LR-N-12945 (CGACCTC GATGTTGAATTAA) (Hedin & Maddison 2001) as a third nested primer for internal sequencing of these amplifications. We tested the ability of other primers to amplify a shorter fragment (~650 bp) of the same region that could be sequenced in a single reaction. We compared amplifications from LR-N-12945 with NI-J-12261, plus LR-N-13398 with NI-J-12581 (CCTTTACGAATTTGAATATA) (Hedin & Maddison 2001) and Hb16S (TTA CGGAAGTGCACATATCG) with HbND1 (TGAGCTACTCTTCGAATAGC) (Masta 2000a).

To amplify ~1-Kb of the mitochondrial Cytochrome Oxidase (CO1) gene, we used primer C1-J-1751 (GAGCTCCTGATATAGCTTT TCC) with C1-N-2776 (GGATAATCAGAA TATCGTCGAGG) (Hedin & Maddison 2001). Fragments were amplified using a thermal cycle of 95° (120 s); 35 cycles of 95° (30

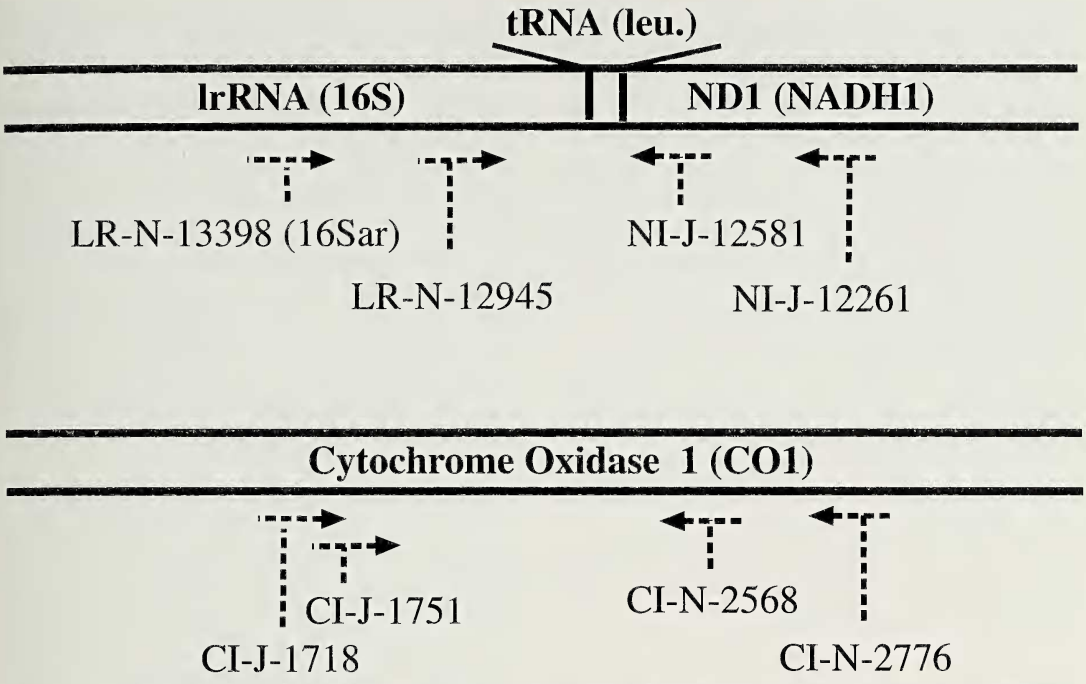

Fig. 1.—Top: The lrRNA-ND1 fragment of the mitochondrial genome sequenced in *Brachypelma vagans* (adapted from Masta 2000b). Bottom: The CO1 mitochondrial fragment sequenced in *B. vagans*.

s), 52° (30 s), 72° (110 s); and 72° (10 min), modified from Hedin (2001) and Hedin & Maddison (2001). We used CI-N-2568 (GCT ACAACATAATAAGTATCATG) (Hedin & Maddison 2001) as a nested primer for sequencing (See Fig. 1, bottom). Again, we tested other primer combinations for amplification consistency including C1-J-1751 with CI-N-2568 (GCTACAACATAATAAGTATC ATG) (Hedin & Maddison 2001) and CI-J-1718 (GGATCACCTGATATAGCATTCCC) (Simon et al. 1994) with CI-N-2776. Finally, to amplify ~350 bp of the nuclear (rRNA) internal transcribed spacer 2 (ITS-2), we used the primers 5.8S (GGGACGATGAAGAACG CAGC) with 28S (TCCTCCGCTTATTGAT ATGC) (Hedin 1997b). Reactions were run using 95° (120 s); 35 cycles of 95° (30 s), 48° (60 s), 72° (110 s); and 72° (10 min).

Purification and Sequencing.—PCR products were purified using a Gene-clean II kit (Bio 101), and run on an ABI PRISM® 377 automated sequencer (PE Applied Biosystems) using standard protocols. We sequenced both strands of the largest reliable amplification products for all specimens (except ITS-2 from Cuevas 10 and 11). Chromatograms were edited using Sequencher™ 4.1 (Gene Codes Corp, MI), and ambiguities scored with IUPAC coding. Sequences of *B. vagans* were deposited in GenBank as AJ585053–AJ585072 (ITS-2), AJ584615–AJ584636 (CO1) and AJ585387–AJ585408 (lrRNA-ND1). For *B. angustum*, the accessions are AJ585073 (ITS-2), AJ584637 (CO1), and AJ585409 (lrRNA-ND1).

Population diversity analyses.—Population diversity was calculated using DnaSP version 3.50 (Rozas & Rozas 1999) assuming that the lrRNA-ND1 and CO1 mitochondrial fragments show uni-parental inheritance and complete vegetative assortment, while nuclear ITS-2 is autosomal and diploid (though probably part of a repetitive family). For each fragment (lrRNA-ND1; CO1 and ITS-2) we evaluated variation within each population by the number of segregating nucleotides per sequence (S), and average nucleotide differences (κ) (Nei 1987). Values for S and κ were used to estimate the neutral parameter (θ) (Watterson 1975; Tajima 1983), and the D statistic of Tajima (1989). Under the finite-sites model, the differences between estimates of θ (from either S or κ) are expected to be zero where there is no selection and demographic conditions are at equilibrium. Population subdivi-

sion was described using the F_{ST} statistic (Wright 1951), which estimates the fraction of sequence diversity attributed to differences between populations (here, colonies). Using the pair-wise variance approach (Hudson et al. 1992), F_{ST} was estimated as 1 minus the ratio of within population heterozygosity (H_w) to between population heterozygosity (H_B). Slatkin & Maddison's (1989) coalescent approach provided estimates of the effective number of migrating individuals per generation (N_m), assuming the sampled genes are selectively equivalent, and that population structure approximates an island model. For each genetic fragment, haplotype relationships were generated using statistical parsimony (Templeton et al. 1995) using TCS version 1.13 (Clement et al. 2000). Other comparisons between sequences of *B. vagans* and other arachnids were made with Blast tools at http://www.ncbi.nlm.nih.gov/BLAST/.

RESULTS

Non-lethal DNA sampling in *Brachypelma*.—Trials of leg autospasy on several captive bred theraphosids (mostly *Brachypelma* spp.) showed that spiders regained normal behavior within 4–5 h, and accepted prey items within a few days (Longhorn 2001). These results matched our field experiences with *B. vagans* in Belize, where we successfully induced autospasy from all spiders, averaging between 30 s and 3 min per specimen. In many cases, juveniles were noticeably more willing to cast limbs than adults. In captive spiders, survival rates were high. At 3 mo post-autospasy, all captive specimens remained alive, although after 6 mo survivorship had decreased to 97% and fell to 94% after 1 yr. Though we did not maintain a separate control sample, this mortality rate is not much different from the standard survivorship of captive tarantulas. However, all surviving spiders in the captive group regenerated lost limb(s) over successive molts. Return observations of the Las Cuevas population during 2004 (by A. P. Vogler) showed a high density of *B. vagans* at this field site three years after autospasy was conducted. During the 2004 re-evaluation, large specimens of *B. vagans* (age > 5 yr) were found to occupy the same burrows where spiders had been returned after tissue sampling. In our opinion, the presence of these large spiders probably indicates the long-term survival of wild caught specimens following autospasy (for DNA), or at the least, re-colonization of empty burrows.

Survey of PCR primers for *Brachypelma*.—We generated ~2200 bp of DNA sequence from 22 *B. vagans*, for three genetic regions (except ITS-2 in Las Cuevas 10 and 11). For lrRNA-ND1, the most consistent PCR amplification was using primers LR-N-13398 and NI-J-12261 to give a 940 bp fragment. However, other primer combinations were quite effective in amplifying a shorter fragment of the same region, like LR-N-12945 with NI-J-12261 which readily yielded ~650 bp. Using the same thermal profile (on request), LR-N-13398 and NI-J-12581 amplified a similar size fragment (~650 bp) with far less consistency and lower product yield than other primer combinations. Primers Hb16S with HbND1 failed to give consistent amplifications in *B. vagans* using the thermal profile for Salticidae (Masta 2000b) or with profile variations. For the CO1, the primer C1-J-1751 worked best with CI-N-2776 to give a 960 bp fragment, but C1-J-1751 also worked well with CI-N-2568 yielding about 850 bp. Here, consistency was increased by adding 5 low stringency cycles of 95° (30 s), 50° (30 s), 72° (60 s) prior to 35 cycles as for CI-J-1751 with CI-N-2776. The same modified profile can also be used to amplify CO1 from other species of *Brachypelma* and more distantly related Theraphosidae (Longhorn 2001, unpublished data). Primer CI-J-1718 with CI-N-2776 failed to give strong amplifications for *B. vagans* under a variety of thermal conditions.

Characterization of the lrRNA-ND1.—The 940 bp fragment of lrRNA-ND1 from *B. vagans* gave the closest nucleotide match (by BlastN) to published mitochondrial sequences from jumping spiders (Araneae, Salticidae). Nucleotide identity was higher between *B. vagans* and several Salticidae (Top match 6e-44 to AY477266; Hedin & Maddison 2001, 2003) than another tarantula (6e-41) *Haplopelma huwenum* (Wang et al. 1993; Qiu et al. 2005) [In Genbank as *Ornithoctonus huwena* NC_005925, but recently transferred to *Haplopelma* (Araneae, Theraphosidae)]. However, measures of similarity alone often give misleading views of taxon affinities, while informative characters can be more useful. That said, similarity is often useful to identify func-

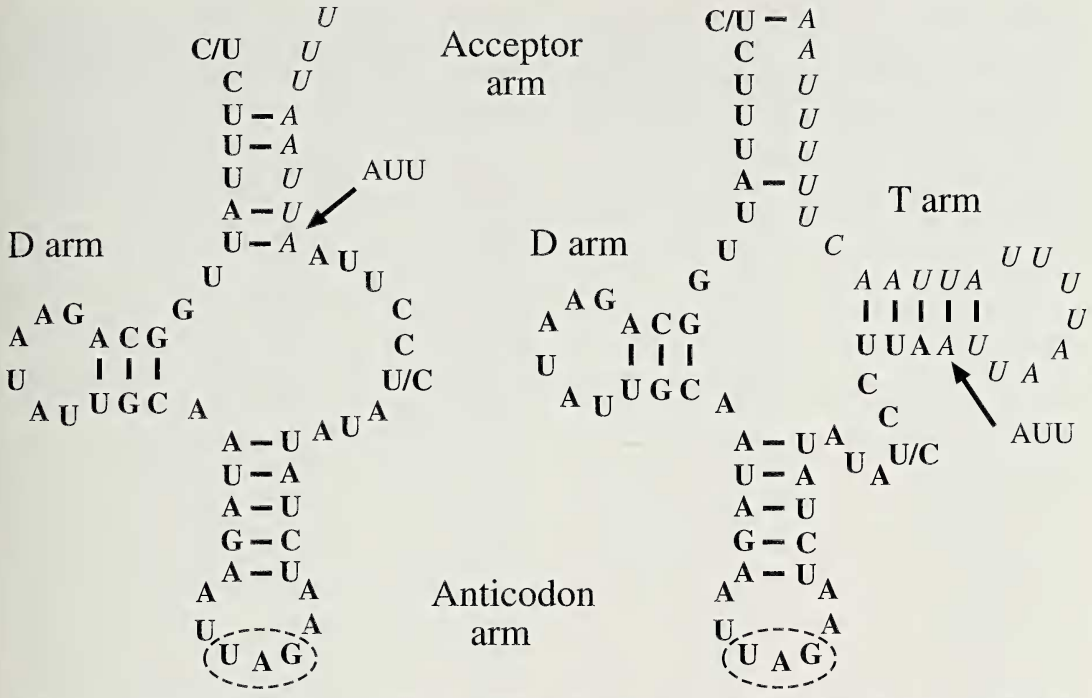

Fig. 2.—Putative secondary structures for tRNA leucine in the mygalomorph spider *B. vagans*. Left: Structure fitted to the truncated model from the araneomorph spider *Habronattus* (Masta & Boore 2004). Right: Fitted to yield a conventional "cloverleaf" structure with a functional TψC arm.

tionally important domains. Four regions of high identity were detected between lrRNA-ND1 of *B. vagans* and other spiders. The largest high identity segment (about 203 nucleotides) corresponded to the peptidyl transferase centre of the lrRNA, identified using the secondary structure map of the salticid *Habronattus oregonensis* (Peckham & Peckham 1888) (Masta 2000b). The next largest identity region (30/33 bp) was the DHU and anticodon arms of $tRNA^{LEU(CUN)}$. With the exception of two sites, the *B. vagans* tRNA sequences were identical across all 22 individuals, and had a higher AT composition (75.6%) than lrRNA (~68.5%). The $tRNA^{LEU(CUN)}$ in *H. oregonensis* folds to an unusual truncated structure, lacking the TψC arm (Masta 2000b; Masta & Boore 2004). According to this truncated model, the TψC arm is substituted by a TV-replacement loop. It is possible to fit a cloverleaf structure to this tRNA in *H. oregonensis*, but this model allows less complementary bases in the acceptor arm than the truncated model and more problematic overlap with ND1 (Masta 2000b; Masta & Boore 2004). The truncated model fits reasonably well to $tRNA^{LEU(CUN)}$ from *B. vagans*, with strong intra-molecular pairing (high degree of complementary) in the DHU-arm (Fig. 2, left). In the anticodon arm, the UAG anticodon was found as expected, supported by five paired stem bases as *H. oregonensis*. The cloverleaf structure fits well to the *B. vagans* data (Fig. 2, right), revealing the possibility of a conventional TψC arm. For our data, the cloverleaf model also requires greater mismatch in the acceptor stem than the truncated model plus more overlap with ND1 (Longhorn 2001). The start codon of *Brachypelma* ND1 appears to be an atypical ATT (AUU), as in other spiders (Masta 2000b). To accept the cloverleaf structure as the preferred model for *B. vagans* requires the first seven codons (18 b.p.) of ND1 to also function as part of the $tRNA^{LEU(CUN)}$. If allowed, our data suggest that the canonical cloverleaf model provides a better fit to *Brachypelma* $tRNA^{LEU(CUN)}$ than the truncated model, which fitted best for *H. oregonenesis* (Masta 2000b).

Characterization of ITS-2.—The 358 bp ITS-2 from *B. vagans* gave the closest nucleotide (BlastN) match to *Aphonopelma hentzi*

(Girard 1852) (AY210803, Mallatt et al. 2004). This is another tarantula closely allied to the *Brachypelma*, both in the subfamily Theraphosinae (Smith 1994). A single region of high identity (e-129) was found between these two genera (92%; 338/367). Across different *B. vagans*, much of the fragment at the 5.8S rRNA end is invariant (up to 120 bp here; 162 bp in *A. hentzi*). The small segment of 28S rRNA included was also invariant across *B. vagans*, with only two nucleotide differences from *A. hentzi*. In the actual ITS-2 spacer, there were size differences between *A. hentzi* (203 bp) and *B. vagans* (193 bp) and nineteen inter-specific nucleotide differences, most at the 3′. The available ITS-2 from other spiders matched *B. vagans* with much less identity, the next most significant (2e-50) was *Orsonwelles* spp. Hormiga 2002 (Araneae, Linyphiidae) (Hormiga et al. 2003). Sequences from other araneomorphs matched less well again, like Theridiidae (S.W. A'Hara, unpublished), Nesticidae (Hedin 1997b), Linyphiidae (Hormiga et al. 2003) and Salticidae (Arnedo & Gillespie 2006), many of which had a similar size (150–250 bp) to our ITS-2 (193 bp), considerably smaller than other arthropods.

Characterization of CO1.—The 960 bp fragment of *B. vagans* CO1 gave the closest nucleotide (BlastN) match to *Promyrmekiaphila* sp. Schenkel 1950 (Araneae, Mygalomorphae, Cyrtaucheniidae: AY621508; Bond 2004) with 84% identity (2e-151, 623/740 sites). Similarly, CO1 from other mygalomorphs also matched *B. vagans* with a high identity. For example, CO1 from *Sphodros abbotti* Walckenaer 1835 (Atypidae: AF303528; Hedin 2001) matched with 83% nucleotide identity (559/666 sites), while *Apomastus schlingeri* Bond & Opell 2002 (Cyrtaucheniidae: e.g., DQ388588; Bond et al. 2006), or *Antrodiaetus unicolor* (Hentz 1842) (Antrodiaetidae: e.g., AY896899; Hendrixson & Bond 2005) matched with higher identity (up to 85%) but over a shorter length (up to 534/622 sites). The most significant match (6e-124) to another member of the family Theraphosidae was with *H. huwenum* (Qiu et al. 2005; as *O. huwena*). Surprisingly, the level of CO1 identity between these tarantulas was almost identical to levels seen between *B. vagans* and other families of mygalomorph spiders (83%; 518/616 sites, versus above). This was surprising as *Brachypelma* and *Haplopelma* only currently warrant different subfamilies (both Theraphosidae, Theraphosinae and Ornithoctoninae, respectively).

Recently, a few CO1 sequences from other *Brachypelma* species have been published (Petersen et al. 2006). Each of these is quite short, only averaging around 300 bp, and severely limits the number of sites for comparison. The highest identity was between our *B. vagans* and *B. albopilosum* up to 97%; (DQ224243, 141/144 sites), followed by *B. angustum* (to 95%, DQ224245, 137/144). This result supports a close phylogenetic position of *B. vagans* with these Mesoamerican species, compared to other *Brachypelma* from the Pacific coast of Mexico, like *B. smithi* (as in Longhorn 2001; see also Petersen et al. 2006). Translated (BlastX) searches showed that *B. vagans* sequences also displayed greatest amino acid similarity with *H. huwenum* (85%; 268/312 sites). However, the next most significant matches were from araneomorph spiders of the Salticidae, probably due to convergence. Overall, the *B. vagans* CO1 showed similar nucleotide composition to other spiders except *Heptathela* (Table 3), which suggested that shared biases in composition were not a factor that led protein searches to identify high similarity with between salticids and *B. vagans* or the unexpectedly high genetic divergence between *Brachypelma* and *Haplopelma* CO1 sequences.

Population structure in *Brachypelma vagans*.—It was possible to join all 22 *B. vagans* sequences by parsimonious connections into a haplotype network for each fragment (Fig. 3), each identical with gaps were coded as missing or 5th state. The two mitochondrial fragments (lrRNA-ND1 and CO1) gave similar structure among individuals and separated the two populations (Pooks Hill and Las Cuevas). The lrRNA-ND1 (Fig. 3, top) revealed slightly more divergence between populations than CO1 (Fig. 3, middle) though both regions are size equivalent (~950 bp). Both mitochondrial fragments also revealed more unique haplotypes in the Las Cuevas population than at Pooks Hill, and both separated the outgroup *B. angustum*. These results contrast with the smaller ITS-2 (Fig. 3, bottom), which could not distinguish the two *B. vagans* populations, nor showed sufficient nucleotide differences to separate the outgroup. Across all fragments,

Table 1.—Specimens with GenBank accession numbers. Samples of *B. vagans* are listed by their collection location, either from P = Pooks Hill or C = Las Cuevas.

Sample (all *B. vagans* unless indicated)	Carapace Length (cm)	Fragment sequenced		
		ITS-2	CO1	lrRNA-ND1
P1	1.95	AJ585053	AJ584615	AJ585387
P2	2.20	AJ585054	AJ584616	AJ585388
P3	2.00	AJ585055	AJ584617	AJ585389
P4	2.50	AJ585056	AJ584618	AJ585390
P5	3.00	AJ585057	AJ584619	AJ585391
P6	2.70	AJ585058	AJ584620	AJ585392
P7	2.20	AJ585059	AJ584621	AJ585393
P8	2.85	AJ585060	AJ584622	AJ585394
C9	2.20	AJ585061	AJ584623	AJ585395
C10	3.00	No Data	AJ584624	AJ585396
C11	3.00	No Data	AJ584625	AJ585397
C12	1.80	AJ585062	AJ584626	AJ585398
C13	1.50	AJ585063	AJ584627	AJ585399
C14	1.90	AJ585064	AJ584628	AJ585400
C15	1.75	AJ585065	AJ584629	AJ585401
C16	3.10	AJ585066	AJ584630	AJ585402
C17	2.50	AJ585067	AJ584631	AJ585403
C18	1.40	AJ585068	AJ584632	AJ585404
C19	1.70	AJ585069	AJ584633	AJ585405
C20	1.40	AJ585070	AJ584634	AJ585406
C21	3.00	AJ585071	AJ584635	AJ585407
C22	2.40	AJ585072	AJ584636	AJ585408
B. angustum	1.75	AJ585073	AJ584637	AJ585409

there were thirty-six polymorphic nucleotides, twenty-four of which were parsimony informative (lrRNA-ND1 = 11 sites [1.14%] > CO1 = 10 sites [1.06%] > ITS-2 = 3 sites [0.84%]). Details of nucleotide polymorphism within and between populations of *B. vagans* are given in Tables 4 and 5. In both populations, the number of segregating nucleotides per sequence (S) was similar (except with lrRNA-ND1). The Pooks Hill population consistently had a greater number of average sequence differences (κ) among individuals than Las Cuevas, despite fewer haplotypes overall. Within each population, our results suggest that differences in directional selection are not detectable (neither population deviates significantly from neutrality) and that demographic conditions are relatively stable (non-significant Tajima's D statistic).

Table 2.—Mean nucleotide composition of genetic fragments of *B. vagans*.

Fragment	Length (bp)	Adenine (A)	Cytosine (C)	Guanine (G)	A + T (%)
CO1	963	0.206	0.121	0.229	65.0
lrRNA rRNA	498	0.349	0.156	0.159	68.5
$tRNA^{(LEU)}$	53	0.377	0.131	0.113	75.6
ND1	396	0.331	0.174	0.110	71.5
5.8S rRNA	119	0.197	0.267	0.309	42.4
ITS-2	193	0.187	0.294	0.296	41.0
28S rRNA	45	0.330	0.252	0.209	53.9

For both the CO1 and lrRNA-ND1 fragments, the largest proportion of sequence diversity was attributable to differences between populations. Estimates of gene flow and population structure were difficult to determine with ITS-2. This was surprising, as this region is often large and variable in arthropods, and hence widely used for phylogeographic studies. Estimates of between population gene flow between using F_{ST} values were much lower from nuclear ITS-2 than from either mitochondrial fragment, reflecting its short length and relative invariance. Overall, results

Table 3.—Nucleotide composition of CO1 across exemplar Araneae. * = Complete.

[Infraorder] Family	Genus	Accession	% A	% C	% G	Length	A + T
[Aran.] Agelenidae	*Tegenaria*	AY138836	22.7	15.3	19.8	450	64.9
[Aran.] Araneidae	*Argiope*	AY731171	27.5	12.1	18.8	1536*	69.1
[Aran.] Desidae	*Badumna*	AF218280	25.4	11.2	20.7	552	68.1
[Aran.] Eresidae	*Stegodyphus*	AY611805	26.6	11.1	19.7	1000	69.2
[Aran.] Linyphiidae	*Frontinella*	DQ029220	23.8	13.0	19.5	954	67.5
[Aran.] Lycosidae	*Rabidosa*	DQ029232	23.9	11.6	20.4	942	68.0
[Aran.] Oecobiidae	*Uroctea*	DQ973166	22.8	13.2	21.2	964	65.7
[Aran.] Salticidae	*Habronattus*	NC005942	26.8	11.4	18.7	1542*	69.9
[Aran.] Thomisidae	*Xysticus*	AY297423	24.8	12.7	17.9	1047	69.4
[Aran.] Tetragnathidae	*Nephila*	NC008063	27.1	11.7	18.7	1536	69.7
[Aran.] Dysderidae	*Dysdera*	AF244321	21.2	14.0	22.9	471	63.1
[Aran.] Hypochilidae	*Hypochilus*	AF303527	23.3	14.0	22.4	1536	63.6
[Mygal.] Antrodiaetidae	*Antrodiaetus*	AY297423	23.7	11.4	21.0	1008	67.6
[Mygal.] Atypidae	*Sphodros*	AF303528	21.3	11.9	21.2	1047	66.9
[Mygal.] Cyrtaucheniidae	*Apomastus*	DQ389886	25.7	11.6	19.0	810	69.4
[Mygal] Hexathelidae	*Atrax*	AAL11676	25.4	12.9	19.6	658	67.5
[Mygal.] Theraphosidae	*Ornithoctonus*	NC_005925	24.7	12.2	21.4	1536*	66.4
[Mygal.] Theraphosidae	*Brachypelma*	AJ584636	20.7	12.2	22.9	963	64.9
[Suborder Mesothelae] Heptathelidae	*Heptathela*	NC005924	28.2	17.4	15.1	1533*	67.5
Average (all Araneae)			24.5	12.7	20.0	1057	67.3
Average (only Araneomorphae)			24.6	12.5	20.1	1090	67.4
Average (only Mygalomorphae)			24.2	12.8	20.0	1079	67.1

indicated that the Pooks Hill population is more structured than at Las Cuevas, even though fewer individuals were sampled at Pooks Hill. An equally plausible explanation is that the Pooks Hill population is older (under neutrality). Estimates of the effective numbers of migrating individuals per generation (Nm) were surprisingly low from both mitochondrial fragments, almost to a level where it is difficult to distinguish between low and non-existent levels of gene flow among populations, suggesting a high degree of mitochondrial sequence isolation.

DISCUSSION

Non-lethal DNA sampling by autospasy.—The evaluation of non-lethal DNA sampling techniques is important in any genetic studies where the goal is conservation. Here, we induced limb autospasy from fossorial tarantulas for genetic material. We refer to this induced response as autospasy rather than autotomy, which has been incorrectly used elsewhere and strictly applies to a reflex action alone (after Piéron 1907; Wood 1926; Roth & Roth 1984). In general, the ability to cast limbs is found in a wide variety of arthropods. In most spiders, limb separation involves rupture at the coxa-trochanter boundary, achieved by snapping the coxa upwards while the femur is kept static (Bauer 1972). This is followed by muscle contraction around the internal margin of the coxa to close the wound and minimize hemolymph loss (after Wood 1926; Bauer 1972). In some cases, autospasy can occur at the patella-tibial joints in certain long-legged Linyphiidae and perhaps Filistatidae (Roth & Roth 1984). A third type of autospasy has been described between the femur and patella, at the patellar cleavage plane, but only been in two genera of Agelenidae (Roth 1981). Autospasy can be easily induced in other arachnids, especially Opiliones, but not in Scorpiones (Wood 1926) or some primitive spiders (Roth 1981). Therefore, while limb removal is probably a useful method to obtain non-lethal DNA samples from most spiders, it is not universally applicable for all arachnids.

To reduce trauma to *B. vagans* during tissue sampling, we considered CO_2 anesthetization, as used to attach radio-transmitters (Janowski-Bell & Horner 1999) or insert transponders (Reichling & Tabaka 2001). However, because autospasy is partly voluntary, we suggest that

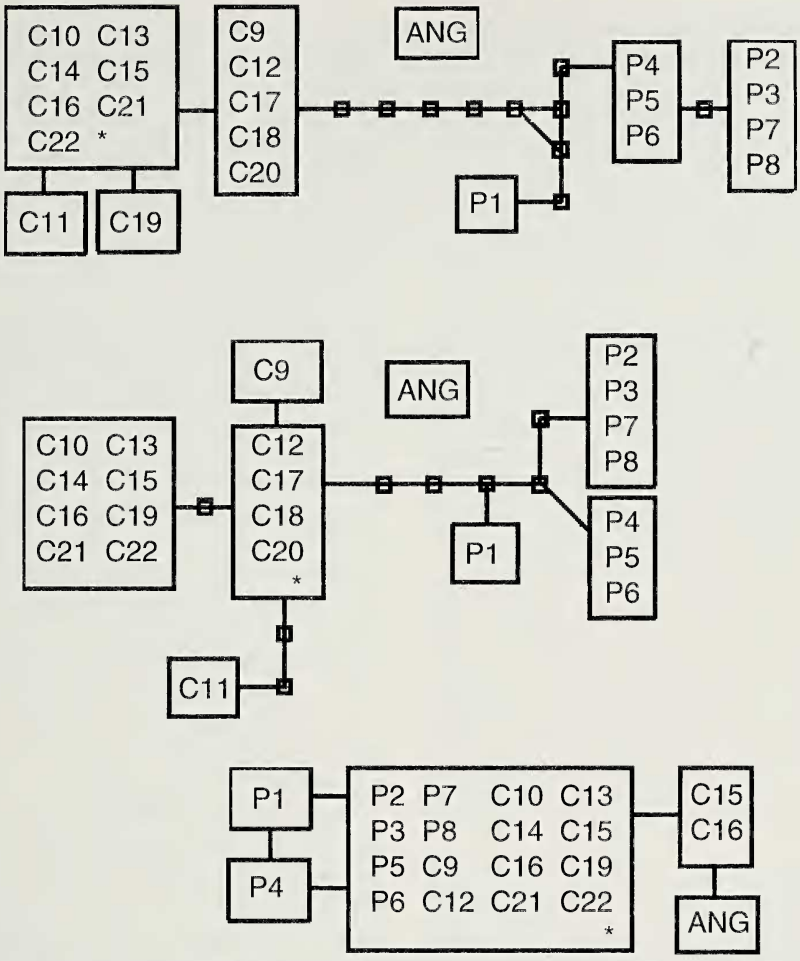

Figure 3.—Haplotype networks for the two *B. vagans* populations (top = from lrRNA-ND1; middle = CO1; bottom = ITS-2). In all cases gaps = missing data. Asterisked boxes notify ancestral sequences. Black lines represent single mutational steps, and small squares lost/un-sampled haplotypes. P = Pooks Hill population of *B. vagans*, C = Las Cuevas population of *B. vagans*, and ANG = *B. angustum*.

the method would be damaging to anesthetized specimens and result in excessive fluid loss (as shown by Bonnet 1930). Without anesthetization, one captive tarantula did show more hemolymph loss than other similar sized spiders after autospasy, either in captive or field populations. Even after the application of artificial coagulants (corn starch and nail

Table 4.—DNA polymorphism within populations.

Gene	Population	Haplo-types	(S)	Nucleotide differences (κ) -gene	θ (S) -gene	Tajimas' D	Significance
COI	Las Cuevas	4	6	1.626	1.887	−0.495	> 0.1 = N/s
	Pooks Hill	3	6	2.428	2.314	0.231	> 0.1 = N/s
lrRNA-ND1	Las Cuevas	4	3	0.780	0.943	−0.529	> 0.1 = N/s
	Pooks Hill	3	7	2.393	2.699	−0.541	> 0.1 = N/s
ITS-2	Las Cuevas	2	1	0.303	0.331	−0.195	> 0.1 = N/s
	Pooks Hill	2	1	0.250	0.385	−1.055	> 0.1 = N/s

Table 5.—DNA polymorphism between populations.

Gene	Fixed nucleotide differences	Avg. nucleotide differences (K) between populations	F_{ST}	Nm
COI	3	7.679	0.736	0.18
lrRNA-ND1	6	9.786	0.838	0.10
ITS-2	0	0.519	0.520	4.56

hardener) the wound re-opened when this spider moved, though fluid-loss finally ceased when it was artificially restrained. Closer examination showed abdominal darkening characteristic of the pre-molt phase, and the spider successfully molted two weeks later. The molt occurred without any regeneration of the lost leg, which was not observed until a subsequent molt (8 mo later). As tarantulas are unwilling to accept prey items during their molting period, linking forced autospasy with prey-lure collection techniques may lower the probability of capturing pre-molt individuals in field studies, and prevent this potential problem. However, if needed, tarantulas can willingly cast limbs while in pre-molt, though this may be the most vulnerable time for limb removal.

Alternative strategies for genetic studies of *Brachypelma*.—DNA can be easily extracted from exuviae (Peterson et al. 2007), which provides an alternative non-lethal tissue source than limb removal for arthropods. While DNA yields are low, it is possible to amplify high-copy fragments (like lrRNA). However, current attempts to use tarantula exuviae have either failed to sequence large fragments or been confounded by fungal contaminants (Longhorn 2001; Peterson et al. 2007). With more taxon specific primers, exuviae will doubtless provide a useful, albeit challenging tissue source for DNA samples, especially for captive spiders. That said, exuviae are little used in genetic studies of natural populations, mainly due to DNA degradation and contamination. Furthermore, most adult tarantulas molt yearly (or less), which is unlikely to coincide with a short research period and these spiders often keep old exuviae deep inside their burrows, making it difficult for the investigator to access without undue damage. The most valuable use for exuviae may be for genetic investigations of tarantulas seized during wildlife enforcement (Peterson et al. 2007), although investigators must again wait for a molt, probably an intolerable delay. Regardless of approach, it is critical to promote non-lethal DNA sources in conservation-focused studies, and build a resource of characterized genetic data. For the genus *Brachypelma*, a DNA reference collection may be extremely useful to wildlife-trade enforcement and conservation efforts. At best, such a genetic resource would be based on samples with clear provenance and a supporting morphological determination. Our genetic samples of *B. vagans* fulfil these criteria for this species and provide a useful reference for comparison with similar spiders of uncertain history or collection location.

Population structure in *Brachypelma*.—Due to the small number of genetic fragments sampled, it is unknown whether the discrepancies in F_{ST} between the three genetic regions can be attributed to differences between nuclear and mitochondrial gene flow in general. However, the two-mitochondrial fragments did not suggest differential selection pressures. Reasonable levels of polymorphism diversity in these two fragments allowed gene flow estimates (Nm) to be calculated with more confidence than from the less variable nuclear ITS-2. However, the validity of gene flow estimates based on indirect measures such as F_{ST} is unclear. Slatkin (1989) suggested that when samples are large (around 10 or more individuals, as here), values for Nm are robust estimators of gene flow. More recently, it has been proposed that measures of genetic variability based on F_{ST} may be valuable, but their transformation into quantitative estimates of gene flow may be unnecessary at best, and misleading at worst (Whitlock & McCauley 1999). Theoretical concerns aside, estimates of gene flow are important to understand species integrity. In general, gene flow acts to counter the effects of isolation. Broadly, gene flow influences the persistence

of local populations and facilitates the spread of adaptive traits among complex landscapes (Hanski & Gilpin 1997). Direct measures of migration may be the most preferable approach to estimate gene flow, but such measures have only been applied cursorily in tarantulas with implanted tags (Reichling & Tabaka 2001) or radio-transmitters (Janowski-Bell & Horner 1999). As well as the time-constraints and technological difficulties, direct measures of dispersal may not reflect the actual movement of genetic material. For gene flow to have occurred, the migrant must also reproduce effectively in the new location. This is probably not the case for the majority of male tarantulas, which can fall prey to predators, harsh environmental factors, or even unreceptive females.

Indirect estimation of gene flow in tarantulas.—The estimation of gene flow in fossorial tarantulas is complicated by the influence of sex-biased dispersal. In all species of *Brachypelma*, mature females display strong site fidelity while mature males show long-range dispersal. Due to these sex-specific aspects, estimates of population subdivision from maternally inherited mitochondrial DNA can be over-estimates compared to bi-parentally inherited nuclear DNA (Gómez-Zurita & Vogler 2003). In fossorial tarantulas, direct measures of gene flow from male migration may provide the best estimates of long-range gene flow, but these overlook finer scale effects of juvenile dispersal and colony formation. The indirect estimation of gene flow from maternally inherited mitochondrial fragments may present the clearest picture of fine sub-structure and gene flow. We concede that inferences from mitochondrial markers are probably not suitable to track genetic admixture from long-range male dispersal, but suggest that finer scale genetic differences within populations are equally interesting.

For our samples of *B. vagans*, results are consistent with the Pooks Hill population having more connectivity with neighboring populations than those at Las Cuevas. Field observations agreed as the Pooks Hill population was from a semi-open grassland habitat indistinguishable from nearby regions where it was likely that *B. vagans* also occurred in high densities. In contrast, the Las Cuevas population was restricted to an isolated grass clearing enclosed by moist broadleaf forest where intensive searches failed to reveal further *B. vagans* burrows (but these have since been reported to exist at low densities).

Overall, the geographic distance between the two *B. vagans* populations (about 50 km) reflects the entire range of other, more threatened species in the genus *Brachypelma* (Locht et al. 1999; West 2005). As a result, inferences from our localized geographic sampling of *B. vagans* may provide a useful baseline to compare against the overall genetic divergences from other species of *Brachypelma*. However, ecologies of *B. vagans* slightly differ from other species, and comparisons of divergences across taxa should be treated with caution since different *Brachypelma* species probably differ in their abilities to colonize new areas.

Alternative sources of nuclear genes.—The nuclear ITS-2 was not suitable to resolve genetic sub-structure in *B. vagans* due to its short size and relative invariance. At the time of this study, the only known spider ITS-2 were from the araneomorph families Theridiidae and Nesticidae at ~150–250 bp (Hedin 1997b), reflecting the paucity of genetic regions amenable to study at that time. Aside from ITS-2 and neighboring rRNA, we initially had few other choices of regions to select, given a lack of nuclear data for any spiders, especially Theraphosidae. There has since been a gradual accumulation of PCR targeted nuclear sequences of spiders in public domain, plus several thousand expressed sequence tags (ESTs) from the tarantula *Acanthoscurria gomesiana* Mello-Leitão 1923 (Lorenzini et al. 2006). Together, these new data can provide a foundation for genetic studies of theraphosid spiders. Several studies have suggested that nuclear gene introns are often the most suitable region for population level genetic studies. However, with EST data, the location of introns is often unknown, as most are derived from mature mRNA. Before additional nuclear markers can be derived from spider ESTs, gene-specific primers need to be designed and the location, size and variability of introns identified at the genomic region of interest. Overall, it would be desirable to explore estimates of population sub-structure in fossorial tarantulas using several nuclear fragments. However, this study confirms that selected segments of the mitochondrial genome can alone provide valuable genetic

data for phylogeographic studies of these spiders.

Of the three genetic regions, the mitochondrial lrRNA-ND1 was best suited for the characterization of population subdivision and genetic polymorphism in *B. vagans*. Similar conclusions arose from both lrRNA-ND1 and CO1, which confirmed the suitability of both these mitochondrial markers for inferring population sub-structure. The knowledge of appropriate molecular makers is vital to facilitate future genetic studies with tarantulas. Both mitochondrial fragments were able to distinguish individuals of *B. vagans* from different collection sites, and both regions revealed a greater degree of population sub-structure than anticipated. Such information is critical for conservation, which is often focused around saving as much of the discrete genetic diversity as possible. Taken to the extreme, the spiders from different collection locations could be considered as different phylogenetic species, or at least, discrete geographical lineages, equally worth conservation efforts reflecting their distinct identities.

For the genus *Brachypelma* as a whole, habitat fragmentation continues to threaten the cohesion of natural populations, particularly for the species endemic to the Pacific coast of Mexico. Thankfully, the collection of spiders in the genus *Brachypelma* is now restricted by both national and international laws, including CITES. The next critical step to the conservation of these spiders will be to enhance understanding of the genetic affinities and population sub-structure of each threatened species. At best, future genetic studies of *Brachypelma* will ensure the future survival of viable populations, define species limits, and be used to conserve the maximum genetic diversity of each discrete lineage in this high-profile genus.

ACKNOWLEDGMENTS

We thank the Forestry Department of Belize, and staff at the LCRS, particularly Chris Minty and Chapal Bol. We thank Vicki and Ray Snaddon for their hospitality at Pook's Hill Lodge. This work was funded by a LCRS research grant, the American Tarantula Society, and British Airways "Conservation Scheme." We are indebted to Steve Reichling for field advice, and to Andrew Smith for his faith that genetics may someday be useful in theraphosid systematics. We thank Susan Masta and Jesus Gómez-Zurita for valuable comments, Pierre Paquin for French translation, and Paula Cushing and Gail Stratton for assistance with the manuscript. This work formed part of a Masters thesis (2001) of the first author from Imperial College, London, UK.

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Manuscript received 10 June 2005, revised 25 April 2007.